REMARKS/ARGUMENTS

Claims 16-22 and 32-34 remain in the application.

Objection To The Claims

Claims 20 and 21 have been objected to because "The instant claims encompass non-elected inventions (species) and require amendment to limit to elected invention."

This objection is inappropriate to the extent that it is not authorized by any statute or rule, and is inconsistent with the *Manual Of Patent Examining Procedure* (MPEP). Specifically, the Examiner's attention is directed to MPEP 803.02 (Restriction-Markush Claims), which states in relevant part as follows:

> The provisional election will be given effect in the event that the Markush-type claim should be found not allowable. Following election, the Markush-type claim will be examined fully with respect to the elected species and further to the extent necessary to determine patentability. If the Markush-type claim is not allowable over the prior art, examination will be limited to the Markush-type claim and claims to the elected species, with claims drawn to species patentably distinct from the elected species held withdrawn from further consideration . . . if on examination the elected species is found to be anticipated or rendered obvious by prior art, the Markush-type claim and claims to the elected species shall be rejected, and claims to the non-elected species would be held withdrawn from further consideration. As in the prevailing practice, a second action on the rejected claims would be made final. On the other hand, should no prior art be found that anticipates or renders obvious the elected species, the search of the Markush-type claim will be extended. If prior art is then found that anticipates or renders obvious the Markush-type claim with respect to a non-elected species, the Markush-type claim shall be rejected and claims to the non-elected species held withdrawn from further consideration. The prior art search, however, will not be extended unnecessarily to cover all non-elected species . . . the prior art search will be extended to the extent necessary to determine patentabillity of the Markush-type claim.

Thus, the Examiner does not have authority to require an Applicant to amend a Markush-type claim to delete non-elected species. Instead, the Examiner must (in accordance with the MPEP) extend the search as necessary to determine whether the Markush-type claim is patentable over the prior art.

It is respectfully submitted that the Examiner will agree, after a careful reading of the appropriate sections of the MPEP, that the objection to claims 20 and 21 is improper and should be withdrawn.

Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 16-22 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite.

The Examiner's basis for rejecting claim 16 as indefinite relies on arguments that confuse the distinction between the requirements of 35 U.S.C. §112, first paragraph and 35 U.S.C. §112, second paragraph. More specifically, the Examiner appears to be arguing that the claims are indefinite because they are not enabled. The rejection, in relevant part, reads as follows:

> The claim is indefinite because it is drawn to regenerating the patient's involuted thymus but the steps comprise injecting the immunological equivalent into the regenerated thymus (which means the thymus is already regenerated). Thus, the steps of regenerating the involuted thymus have not been taught. The claim does not set forth any steps involved in the method/process (regenerating an involuted thymus). It is unclear what method/process applicant is intending to encompass. A claim is indefinite where it merely recites a use without any active, positive steps delimiting how this use is actually practiced.

The Examiner has incorrectly stated that the claim is drawn to regenerating the patient's involuted thymus. The claim is directed to a method for transplanting organs and grafting tissue, not a method for regenerating a patient's involuted thymus. Further, the steps do not merely comprise "injecting the immunological equivalent into the regenerated thymus," but instead, first, require a step of "restoring immune system function by regenerating the patient's involuted thymus." Thus, the thymus is not already regenerated. From this complete misapprehension of Applicant's claim 16, the Examiner incorrectly concluded that

the "steps of regenerating the involuted thymus have not been taught." This statement is both incorrect and irrelevant. As demonstrated by the Examiner's own statements at page 5 of the Office Action, methods of thymus regeneration are known in the art, and thus need not be described by Applicant. Further, enablement is immaterial with respect to definiteness. The correct issue under 35 U.S.C. §112, second paragraph is whether those having ordinary skill in the art would understand the metes and bounds of the invention. It is respectfully submitted that those having ordinary skill in the art, as is evident from the prior art of record mentioned by the Examiner, would understand the meaning of the expression "restoring immune system function by regenerating the patient's involuted thymus." Further, although it is inappropriate to base a rejection under 35 U.S.C. §112, second paragraph (indefiniteness) on lack of enablement, the prior art of record also demonstrates that those having ordinary skill in the art know how to restore immune system function by regenerating a patient's involuted thymus.

The Examiner has also rejected the claims under 35 U.S.C. §112, second paragraph, on grounds that the "claim does not set forth any steps involved in the method/process (regenerating an involuted thymus)." This is intentional! Applicant regards his invention to be a "method for transplanting organs and grafting tissue into a patient," not a method of restoring immune function by regenerating a patient's involuted thymus. It is not necessary to limit the method of transplanting organs and grafting tissue into a patient by using any particular step of restoring immune system function by regenerating the patient's involuted thymus. Particular techniques for restoring immune function are the subject matter of dependent claims 20-23. There is not any statute or rule that authorizes an Examiner to arbitrarily demand that an Applicant incorporate limitations into a claim. More specifically, 35 U.S.C. §112, second paragraph, does not provide any authorization for rejecting claims as being too broad. Applicant has intentionally encompassed methods for transplanting organs using any step of restoring immune system function by regenerating the patient's involuted thymus, injecting the immunological equivalent of the tissue or organ to be transplanted into the patient into the regenerated thymus, and then transplanting the organ. This would be clear to those having ordinary skill in the art. Accordingly, the requirements of 35 U.S.C. §112, second paragraph have been met.

The Examiner has further stated that "a claim is indefinite where it merely recites a use without any active, positive steps delimiting how this use is actually practiced." Applicant agrees. However, claim 16 recites active, positive steps delimiting how the method of transplanting organs is actually practiced. These steps include restoring immune system function, injecting the immunological equivalent of the tissue or organ to be transplanted into the patient into the regenerated thymus, and then transplanting said organ.

The Examiner has further stated that claim 16 is "indefinite because the term 'immunological equivalent' is a relative term . . . not defined by the claim . . ." First, there is not any statute or rule that requires an Applicant to define every term that is used in a claim. It is only necessary that those having ordinary skill in the art can understand the metes and bounds of the claims based on the ordinary meanings of the words, and any definitions provided in the specification. The expression "immunologically equivalent" is frequently used in the literature, and means that the immunologically equivalent material induces an immunological effect equivalent to a specific material, e.g., the tissue or organ to be transplanted into a patient. Further, contrary to the Examiner's statement, the meaning of the expression "immunological equivalent" is defined in the specification (at pages 15, line 24 through page 16, line 4), which states that donor-specific cells or antigens that are the immunological equivalent of the tissue itself are materials that stimulate "deletion or anergy of the cells otherwise responsible for later rejecting the transplanted tissue or organ," and include "endogenously-derived sample in the case of those with autoimmune diseases . . .." Those having ordinary skill in the art have been adequately informed and would have been fully aware of what types of material constitute immunological equivalent materials. Thus, the requirements of 35 U.S.C. §112, second paragraph have been met by the claims.

The Examiner has correctly noted that claims 17 and 18 recite the limitation "intrathymic injection" without providing adequate antecedent basis. The Examiner has further correctly noted that claim 19 is indefinite because it provides an example. Finally, the Examiner has correctly noted that claims 20, 21, and 22 recite the limitation "step of regenerating" without providing adequate antecedent basis. It is respectfully submitted that

claims 17-22 have been amended to overcome these bases for rejection under 35 U.S.C. §112, second paragraph.

In view of the above amendments and remarks, it is respectfully submitted that the pending claims comply with the definiteness requirement of 35 U.S.C. §112, second paragraph. Accordingly, Applicant requests withdrawal of the rejection.

Rejection Under 35 U.S.C. §112, First Paragraph

Claims 16-22 and 32-34 have been rejected under 35 U.S.C. §112, first paragraph, on grounds that the claims are not enabled by the specification.

The Examiner has noted that the Greenstein et al. reference demonstrates "that regeneration of an age-involuted thymus can be accomplished in rats using an analogue of luteinizing hormone-releasing hormone (LHRH)," and that the McCormick et al. reference discloses "that regeneration of an age-involuted thymus can be accomplished in rats using growth hormone (GH)." Thus, the Examiner's statements demonstrate that regeneration of an involuted thymus is well known in the art. However, the Examiner has taken the position that regeneration of a patient's involuted thymus does not necessarily result in restoration of immune system function. This is apparently based on a disclosure in the McCormick et al. reference that "there was no significant improvement of cellular immune function" associated with thymus regeneration using growth hormone, and the disclosure in the Goff et al. reference that a change in thymic morphology does not prove increased or decreased thymic function. It is apparent from the Examiner's statements that the Examiner has doubts, based on the Examiner's understanding of the prior art, as to whether the techniques disclosed by Applicant will actually result in restoration of immune function.

It is respectfully submitted that the Examiner's reliance on the McCormick et al. reference is misguided.

McCormick treated 24 month-old mice. Mice of this age are at the mean lifespan for their species, meaning that one-half of 24 month-old mice have already died of aging before treatment with growth hormone has begun. This would translate to a human being in his or her 70s, which is not an age that is likely to qualify for an organ transplant at the present time and therefore that is not representative of the majority of the population envisioned to be

treated by the instant invention. Furthermore, GH-treated mice in the McCormick study did show significant improvement in basal Con A response and in Con A stimulated proliferation at 6 microgram/ml of Con A, as demonstrated by Student's t-test, and the authors reporting that "indications that IL-2 production was beginning to improve" and "an improved cellular immune response is beginning to emerge after eight weeks of treatment." Kelley et al. (PNAS 83, pp. 5663-5667, cited on page 2 of the Dec. 3rd, 2001 Information Disclosure Statement by Applicant) had already shown that rats 24 months of age attained substantial but much less robust restoration of immune function than rats that were only 18 months of age (human equivalent, approximately 56, which represents a prime candidate for organ transplantation), and that the younger rats also had more complete thymic regeneration. Therefore, the McCormick study needs to be interpreted (as McCormick et al. themselves interpreted it) in the light of Kelley et al. and in the light of numerous other studies, including studies known to the Examiner, showing that thymic regeneration is accompanied by restoration of immune function, although restoration of function occurs at a slower rate at extreme old age. Given this context, McCormick et al. do not in any way contradict the teachings of the instant application. The Examiner also notes the McCormick et al. finding of a high incidence of hepatic tumors in the GH-treated old rats. However, this observation is unique in the literature and does not reflect the vast experience now available with both animal and human GH administration, in which no increased incidence of cancers has been observed.

The Examiner's reliance on the Goff et al. reference is also misguided.

The Examiner has quoted Goff as saying "a change (or lack of change) in thymic morphology does not prove increased or decreased thymic function; immunological or endocrine function must also be assessed." However, the basis of this statement was Goff et als.' unique finding that, although the improved immune function of middle-aged dogs coincided with thymic regeneration as expected, in agreement with all other known information in the scientific and medical literature, immune function improved in old dogs even though thymic morphology did not improve. Goff et als.' data imply that thymic regeneration is certain to be accompanied by improved thymic function, but that improved function cannot be taken as proof of thymic regeneration in the oldest old. Since organ transplantation is not likely in the oldest subjects, and since Fahy describes the use of magnetic

resonance imaging for establishing the presence of thymic regeneration (in "Curing Diabetes with HGH and DHA Coadministration," 4th paragraph, first 3 lines), this ambiguity is not germane to the Fahy invention.

Thus, the Examiner's doubt regarding the operability of the invention and/or scope of enablement is based on an incomplete, and consequently incorrect, understanding of the teachings of the prior art. Neither the McCormick et al. reference nor the Goff et al. reference, when properly interpreted, cast any doubt on the ability to restore immune function by regenerating a patient's involuted thymus.

The Examiner has also stated that the Perico et al. reference "does not conclusively demonstrate that immunosuppressive drugs do not interfere with the functional properties of the thymus." However, if immunosuppressive drugs do indeed interfere with the functional properties of the thymus, this appears to be irrelevant given that Perico's transplants all survived as a result of intrathymic transplantation of donor-specific antigens despite treatment with three different immunosuppressive agents, including the cyclosporin mentioned by the Examiner. Further, as Perico note:

> to definitively rule out the possibility that immunosuppressive drugs interfered with the functional properties of the thymus, we used an alternative strategy to deplete circulating lymphocytes by treating the recipients with a single injection of ALS. . . . administration of ALS has never been associated *in vivo* with morphological evidence of thymus atrophy, these experiments can be taken to indicate that the induction of donor-specific unresponsiveness . . . is unrelated to an effect of the immunosuppressive drugs employed on cortical or medullary thymocytes.

The lack of negative effect of immunosuppression on the success of intrathymic transplantation is further supported by Klatter et al. (Transplantation 60: 1208-1210, 1995; reference enclosed), who found successful induction of tolerance to donor hearts using a combination of antilymphocyte serum and a course of cyclosporin given immediately after heart transplantation.

The Examiner quoted Perico et al. on "the possibility that the mechanism we and others have described only applies to the peculiar immune system of rats." However, Liu et

al. successfully used the intrathymic transplant technique to successfully engraft dogs with allogeneic pancreatic islets (Transpl. Proc. 33: 561, 2001, reference enclosed). Thus, it is certain that intrathymic tolerance induction is a general property of thymuses and is not confined to the rat.

Attached hereto are the following general articles:

1. "Long-Term Maintenance of Donor-Specific Unresponsiveness in NIH Miniature Swine by Intrathymic Inoculation With Allogeneic Islets," *Transplantation Proceedings*, Vol. 29, p. 767 1997, Une, S., Oleksy, B., Ohtsuka, S., Arita, S., Kawahara, T., Shevlin, L., and Mullen, Y.;

2. "Long-Term Survival Following Canine Intrathymic Islet Allograft Transplantation With Short-Course Immunosuppressive Therapy," *Transplantation Proceedings*, Vol. 33, p. 561, 2001; Liu, C., Deng, S., Jiang, K., Liu, W., Kenyon, N., Ricordi, C., Naji, A., Barker, C., and Brayman, K.;

3. "Bovine-to-Porcine Intrathymic Islet Xenotransplantation," *Transplantation Proceedings*, Vol. 29, p. 2061, 1997, Del Guerra, S., Giannarelli, R., Carmellini, M., Coppelli, A., Lupi, R., Tellini, C., Solari, R., Meacci, L., Calabrese, M., Viacava, P., Mosca. F., Navalesi, R. and Marchetti, P.;

4. "Simultaneous Transplantation And Intrathymic Tolerance Induction," Vol. 60, No. 11, pp. 1208-1210, December 15, 1995, Klatter, F. A., Raué, H.-P., Bartels, H. L., Pater, J. M., Groe, H., Nieuwenhuis, P., and Kamping, J.; and

5. "Prolonged Allogeneic and Xenogeneic Microchimerism in Unmatched Primates without Immunosuppression by Intrathymic Implantation of $CD34^+$ Donor Marrow Cells[1]," *Cellular Immunology,* Vol. 181, Article No. C1971194, pp. 127-138, 1997, Allen, M. D., Weyhrich, J., Gaur, L., Akimoto, H., Hall, J., Dalesandro, J., Sai, S., Thomas, R., Nelson, K. A., and Andrews R. G.

These journal articles prove that intrathymic transplantation works in dogs, pigs and primates, and therefore is not limited to rats. The fact that the method works in all species from rats to primates shows that it would be expected to work in humans. Since thymic regeneration has been proven by Applicant to work in humans, and intrathymic transplantation has been shown to work on various species, those having ordinary skill in art would expect the

claimed method to work in humans as disclosed. Accordingly, the disclosure is enabling for the claimed invention. Note that none of the new studies used any particularly innovative approaches over and beyond the techniques disclosed in Applicant's specification.

It is unclear what point the Examiner is trying to make with respect to Odorico et als.' findings, since these findings are not contrary to the teachings of the Fahy invention.

The Examiner stated: "The instant specification fails to demonstrate that a patient can have an involuted thymus regenerated.. . ." However, it is necessary only that the instant specification describe a method whereby an involuted thymus can be regenerated if the method is followed. Regeneration has in fact been demonstrated in a human subject as a result of following the directions given in the instant application. A working Example of the efficacy of the Fahy art for the induction of thymic regeneration in as little as 1 month is appended in a declaration of the inventor, Gregory Fahy. The method used in the Example is identical to the method used to generate the data of Table 1 and Figure 6 in the specification (Experiment 2), and the IGF-1 and DHEA target blood levels disclosed in the specification were exactly achieved in the Example. The specification indicates thymic regeneration will be achieved in 1-3 months, and the Example shows a 92% increase in functioning thymic mass in about 1 month. Therefore, the specification as submitted is in fact fully enabling for the attainment of thymic regeneration in humans.

The Examiner stated that, after thymic regeneration, the specification fails to demonstrate that a patient can "then undergo an intrathymic injection and organ transplant or tissue graft." However, if intrathymic injection is possible in rats and dogs, it is reasonable to assume that it is possible in humans. Further, it is well known in the art that humans can undergo an organ transplant or tissue graft. These conclusions are validated by Klatter et al. (see above) who conclude that (page 1209), "this procedure clearly has clinical potential," which would not be the case if intrathymic injection followed by tissue transplants were impossible in humans.

The Examiner stated "Applicants have not demonstrated that this combined method would be successful or better than the methods known in the art for organ transplant/tissue grafts." However, the fact that the intrathymic transplant method works in species as diverse as rats and dogs puts the burden of proof on the proposition that it cannot work in man.

Clearly Klatter et al., cited above, not only believe the method can work in man, but that it represents a "major breakthrough" because "the obvious solution to the problems associated with the long-term use of systemic immunosuppressants would be the induction of specific unresponsiveness to donor antigens only" (page 1208, col. 1). Further, there is not any evidence that would suggest or imply that the function of a regenerated thymus in a young or middle-aged individual would not be very similar to the function of a young thymus, and therefore the burden of proof would rest upon anyone proposing otherwise. Anyone of ordinary skill in the art would be moved to assume that the Applicant's method will be successful and useful in human subjects. Therefore, the Examiner's assertion that "the subject matter sought to be patented as defined by the claims is not supported by an enabling disclosure" is not supported by any known objective facts indicating that the Applicant's invention, as claimed, cannot work.

The Examiner claims "the specification does not provide guidelines to determine thymic atrophy or involution." This ignores the specification's teachings that magnetic resonance imaging or other scanning methods be used to determine the state of thymic atrophy or involution.

The Examiner claims "the specification fails to teach that a thymus can be regenerated upon administration of human growth hormone and DHEA or human growth hormone and chromium picolinate in a patient." This is exactly what the specification teaches, and the Example appended thereto proves that this teaching is enabling, i.e., following the method taught in the specification produces the claimed results.

The Examiner stated "the disclosure does not provide immunological or endocrine assays or employ experiments such as magnetic resonance imaging or morphology studies which would discern that a thymus has been regenerated." However, this does not mean that the disclosure is not enabling. The Example shows enablement for the case of thymic regeneration.

The Examiner stated "the specification provides no guidance or working examples for intrathymic injection." However, intrathymic injection is known in the art and it is not a purpose of the invention to teach what is already known in the art. The fact that human thymic biopsies and animal intrathymic injection methods are known in the art implies that

similar methods can be used in man and would be a trivial point for any thoracic surgeon.

The Examiner stated "the specification fails to teach or disclose working examples for transplanting an organ or grafting of tissue." The Applicant maintains that such techniques are well known in the art and do not require working examples.

The Examiner stated "the specification does not consider factors such as rejection, age-related thymic involution versus other types of thymic involution, the side effects of immunosuppressants, ALS versus CsA, the high incidence of tumors and other side effects associated with GH." However, all of these problems were in fact unavoidably considered in the specification and have been solved in the cited prior art available at the time the invention was made, or are illusory, as in the case of the hepatic tumors found in one highly unrepresentative rat strain. Since age-related thymic involution is the type that the invention successfully reverses, and since it is the prevalent type, other types are not considered to be germane.

In the light of the above facts, the Examiner's conclusions that much experimentation is necessary to regenerate an involuted thymus (note that in the Example the method worked on the first try), administer an intrathymic injection and transplant an organ or tissue (all prior art not requiring any new inventive steps) overcome "lack of direction/guidance presented in the specification" (refuted above in detail) and absence of working examples (not required to practice the invention successfully), deal with the complex nature of the invention (Applicant considers the invention to be functional as specified), and deal with the alleged unpredictability of intrathymic injections and organ/graft transplants (adequately refuted by successful rat and dog intrathymic pancreatic islet injections, which establish tolerance in both species to subsequent islet transplants), are unfounded. Applicant respectfully submits that the amount of experimentation required to successfully practice the invention is customary and normal, and that the full scope of the invention need not be instantly achieved for the invention to be practiced successfully.

It is respectfully submitted that upon careful consideration of the above remarks and attached declaration, the Examiner will agree that the scope of enablement provided by the specification is commensurate with the scope of the claims, such that the claims are in compliance with the requirements of 35 U.S.C. §112, first paragraph.

CONCLUSION

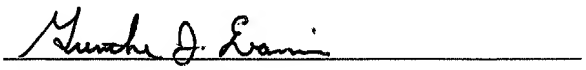
In view of the above amendments, remarks and the attached declaration of the inventor, Dr. Gregory M. Fahy, it is respectfully submitted that the application is in condition for allowance and notice of the same is earnestly solicited.

Respectfully submitted,

GREGORY M. FAHY, PH.D.

By: Price, Heneveld, Cooper, DeWitt & Litton

August 29, 2003
Date



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ELSEVIER

Long-Term Maintenance of Donor-Specific Unresponsiveness in NIH Miniature Swine by Intrathymic Inoculation With Allogeneic Islets

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WE HAVE previously shown that allogeneic islets transplanted into the pig thymus survive for prolonged periods (>200 days) with peritransplant goat anti-pig lymphocyte globulin (ALG) treatment alone,[1] and induce donor-specific unresponsiveness as tested by in vitro assays.[2] In the present study, we investigated the long-term effects of an intrathymic (IT) islet allograft on the recipient's alloimmune responsiveness in vivo.

MATERIALS AND METHODS

Eight NIH minipigs, ranging in age from 5 to 12 months, were injected into either the thymus (IT group, $n = 5$) or the spleen (Sp group, $n = 3$) with islets from a single farm or Yucatan pig donor (24,792 to 102,920 IEQ/pig) with ALG treatment for 5 days. At monthly intervals, until 8 to 11 months, recipient alloimmune responsiveness was tested by various in vitro assays. Delayed-type hypersensitivity (DTH) responses were tested, between 13 and 22 months, by an intracutaneous injection of donor lymphocytes (LC). DTH tests were again performed 2 weeks after the first challenge.

RESULTS AND DISCUSSION

In the IT group: (1) Stimulation indices (SI) of pretransplant mixed-lymphocyte reactions (MLR) were 9.25 ± 1.27 (mean ± SD) against donor LC. Antidonor SI at 1 month posttransplantation dropped to 1.53 ± 0.41 and remained low for >11 months, whereas SI against third-party LC were high in all tests. (2) Recipient peripheral blood lymphocytes (PBL) showed minimal cytotoxic activities against both donor and third-party LC in cell-mediated lymphocytotoxicity (CML) assays. (3) Cytotoxic T-lymphocyte (CTL) activities in MLR-CML against donor LC became undetectable at 1 month, and remained negative for >11 months. The CTL activities against third-party LC were consistently high. (4) At 1 month, precursors of proliferating T lymphocytes (PTL-p) against donor LC decreased to 41% in one pig and to an undetectable level in the remaining three animals. Thereafter, PTL-p were undetectable in all pigs for >11 months. (5) Antidonor cytotoxic antibodies were not detected at any point. (6) All pigs showed no DTH responses in both first and second challenges with donor LC. In contrast, in the Sp group at 1 month: (1) Antidonor SI of MLR increased to 10.98 ± 0.75 from 6.85 ± 0.53. (2) Cytotoxic activities against donor targets increased in both CML and MLC-CML. (3) The PTL-p against donor LC increased. (4) Antidonor antibodies were detectable starting from 1 week. (5) All three pigs showed positive DTH responses (11.0 ± 0.8 mm) upon first challenge, which became greater with the second challenge (18.3 ± 2.1 mm).

These results demonstrate that intrathymic inoculation of islets induces unresponsiveness of PBL as well as suppression of systemic antidonor cytotoxic antibody production and donor-specific DTH responses in vivo. Our results clearly show that an intrathymic islet allograft induces long-term, stable unresponsiveness directed against donor, but not third-party, antigens in a large animal species (swine).

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ELSEVIER

Long-Term Survival Following Canine Intrathymic Islet Allograft Transplantation With Short-Course Immunosuppressive Therapy

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THE THYMUS is the site of T lymphocyte maturation and immune "education," where maturing thymocytes learn to distinguish self (native tissue) from nonself (foreign tissues including invasive organisms). The introduction of specific alloantigen(s) to the thymus may provide a mechanism for the selection of new T cell populations, resulting in recognition of the transplanted alloantigen as self. Intrathymic transplantation of allogeneic pancreatic islets has been successful in small-animal models;[1,2] however, its efficacy in large-animal models has not been clearly defined. To address this question, we used a canine islet transplant model to examine the role of depleting antibody in induction of unresponsiveness following intrathymic islet allografting in the dog.

MATERIALS AND METHODS

In this study, juvenile beagle dogs, 9 to 12 weeks of age, were used as recipients, and the mean body weight was 6.5 ± 0.8 kg. Islets from two donor pancreata were transplanted intrathymically or intravenously (into portal vein) into disparate normoglycemic canine recipients. Recipients were divided into groups of no treatment (control), single treatment (cyclosporine 10 to 20 mg/kg × 3 weeks or anticanine thy.1 antibody 5G2 0.1 mg/kg × 10 days), or dual treatment. 5G2 was one of several monoclonal antibodies generated from fusions between SP2/0 myeloma and splenocytes from Balb/c mice, immunized with canine peripheral blood lymphocytes.[3] Islet recipients underwent total pancreatectomy 3 weeks after transplantation. Islet function was evaluated by serial fasting blood glucose (FBG), intravenous glucose tolerance (IVGTT), and serum insulin measurement; graft survival was examined by conventional and immunohistochemical staining of explanted islet tissue.

RESULTS

After pancreatectomy, nontreated intrathymic islet allograft recipients became hyperglycemic immediately. Histologic analysis revealed little or no islet tissue in the thymus. In the single-treatment groups, islet recipients maintained short-term normoglycemia (3 to 5 days) following pancreatectomy, and similar observations were made in all intraportal transplantation groups (no treatment, single, or combination treatment). In contrast, normoglycemia was achieved for prolonged periods in pancreatectomized intrathymic islet recipients treated with short-term cyclosporine and 5G2 (7, 11, 62, 86, >180, >1.5 year postnative pancreatectomy). The mean survival time for this group was greater than 150 days: immunosuppression was stopped 3 weeks posttransplantation. Results from IVGTT were not significantly different between recipient and naïve animals. Tissue samples harvested from the 11- and 62-day recipients that died normoglycemic due to intersusception and infection, respectively, demonstrated normal islet tissue with no lymphocyte infiltration.

CONCLUSION

This study demonstrates that the thymus is a viable site for islet allografts in dogs. The data show that long-term islet allograft survival can be achieved following intrathymic transplantation combined with initial short-term, depleting immunosuppressive therapy (5G2) and short-term calcineurin inhibition. A state of relative unresponsiveness toward the resident graft emerges long after discontinuation of therapy. Whether prolonged intrathymic residence of allogeneic islet tissue induces a state of donor-specific unresponsiveness toward an extrathymic allotransplant in dogs is currently under investigation.

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ELSEVIER

Bovine-to-Porcine Intrathymic Islet Xenotransplantation

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IT HAS BEEN previously shown that the thymus is a privileged site for islet transplantation.[1–3] Rat islet allografts transplanted into the thymus of temporarily immunosuppressed animals survived indefinitely, and a state of donor-specific unresponsiveness could be achieved.[1] In addition, rat islet xenografts survived long term within the thymus of transiently immunosuppressed mice, and promoted the development of unresponsiveness to donor xenoantigens.[2,3] More recent studies have demonstrated that the thymus may allow the engraftment of autologous and allogeneic islet tissue in dogs and pigs.[4,5] We evaluated whether the thymus may be used as a site of islet transplantation in the discordant bovine-to–porcine islet xenograft model.

MATERIALS AND METHODS

We prepared bovine islets by collagenase digestion and density gradient purification as described previously.[6] The islets were then cultured 2 to 4 weeks before transplantation.[7]

Eight 3-month-old female pigs, weighing approximately 30 kg, received preanesthesia medication consisting of 300 mg ketamine, 0.5 mg atropine, and 2 mg diazepam. A superficial ear vein was then cannulated, and general anesthesia was induced with 70 mg propofol and 150 mg ketamine and maintained with propofol. Under a continuous electrocardiogram (EKG) recording, the skin in the left lateral portion of the neck was cut, the cutaneous muscles were dissected, superficial and deep cervical fascias were entered and the left sternocleidomastoideus muscle was moved laterally. The left cranial portion of the pig thymus was located, and approximately 10,000 bovine islets resuspended in 5 mL of culture medium were injected. At varying time points after islet transplantation, and up to 4 weeks, bovine islet survival was evaluated by hematoxylin-eosin (HE) staining, insulin immunocytochemical (Dako, Milan, Italy) staining, and radioimmunoassay (RIA) measurement of bovine insulin in the pig plasma (after separation of bovine and porcine insulin by reversed-phase high-performance liquid chromatography [HPLC]) in response to intravenous (IV) glucose.

RESULTS AND CONCLUSIONS

Intrathymic islet cells were found within the sheets of thymocytes both at 3 and 7 days from implantation, with well-preserved insulin-containing cells. No islet tissue could be detected after 14 days from transplant. Bovine insulin could be found in the plasma of the transplanted pigs up to 4 weeks from bovine islet transplantation (basal values: 16 ± 2 μU/mL at 1 week, 9.6 ± 0.3 μU/mL at 2 weeks, 10.2 ± 0.8 μU/mL at 4 weeks; peak values: 58 ± 5 μU/mL at 1 week, 28.9 ± 0.2 μU/mL at 2 weeks, 25 ± 4.8 μU/mL at 4 weeks).

We therefore found that transplantation of bovine islets into the thymus of the pig is a feasible and simple procedure, which can permit study of the engraftment of intrathymic islets in this discordant xenograft model. Our results show histologic and/or functional survival of the intrathymic bovine islets up to 4 weeks from implantation. This suggests that also in this discordant, large mammal combination the thymus could be an advantageous site for islet implantation.

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SIMULTANEOUS TRANSPLANTATION AND INTRATHYMIC TOLERANCE INDUCTION

A METHOD WITH CLINICAL POTENTIAL[1]

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It has been shown that donor-specific tolerance to cardiac allografts can be induced by pretreating the prospective recipient with injections of donor splenocytes (intrathymically) and antilymphocyte serum (intraperitoneally) weeks or days before the actual transplantation. This procedure, however, lacks clinical relevance in the case of cadaver donors due to the obligatory interval between the start of the tolerance induction protocol and transplantation. We have tried to devise a protocol in which this interval is eliminated, thus allowing allotransplantation *simultaneously* with tolerance induction.

Our results show that simultaneous cardiac allotransplantation and intrathymic tolerance induction by intrathymic injection of donor splenocytes and treatment with antilymphocyte serum is indeed possible in the PVG to AO high-responder rat strain combination, provided that low doses of cyclosporine are given intramuscularly on day 1, 2, and 3 after transplantation. As we now are able to combine the start of tolerance induction with the actual allotransplantation, this procedure may indeed have clinical potential.

The obvious solution to the problems associated with the long-term use of systemic immunosuppressants would be the induction of specific unresponsiveness to donor antigens only.

A major breakthrough toward this goal was made when Posselt and co-workers (*1*) showed that intrathymic transplanted islets of Langerhans also mediated acceptance of islets transplanted, 120 days later, under the kidney capsule.

Since this discovery, several groups have demonstrated that the interval between tolerance induction and the actual transplantation can be shortened significantly in rat models of cardiac (*2*, *3*), liver (*4*), and renal (*5*) transplantation. However, in the case of cadaver donors, any interval between the tolerance induction and the actual transplantation will prohibit clinical use of the protocol. As such, all these protocols lack clinical potential.

Acceptance of skin grafts in mice can be achieved by 2 injections of antilymphocyte serum (ALS*) on days −1 and and an intrathymic inoculation with splenocytes on day relative to the transplantation (*6*). This protocol also lac clinical relevance due to the ALS injection on day −1.

To develop a method with potential clinical application, have tried to eliminate the interval between the start tolerance induction and the actual allotransplantation.

Recent experiments performed in our laboratory indica that a single intraperitoneal injection of ALS does not elir inate all peripheral T cells (H. Groen, personal communic tion, University of Groningen, 1995). We hypothesized th the remaining lymphocytes were responsible for the rejecti of the allograft when it was transplanted simultaneous with tolerance induction. Consequently, we sought to su press these T cells by using short-term treatment with cycl sporine (CsA) (*7*).

One other group has published a very similar model f heart transplantation in which tolerance was achieved by injections with FK506 on days 0, 2, and 4 following a sing intrathymic inoculation with bone marrow cells simult neously with cardiac allografting (*8*).

Our results show that it is indeed possible to induce acce tance of cardiac allografts in the high-responder PVG to A rat strain combination using this simultaneous transplant tion and intrathymic tolerance induction (STITTI) protoco

MATERIALS AND METHODS

Animals. Age-matched, SPF male AO ($RT1^u$) and female PV ($RT1^c$) rats, 7–10 weeks old, were obtained from the Central Anim Facility of the Medical Faculty of the University of Groningen.

Preparation of donor splenocytes. A single cell suspension of PV splenocytes was prepared by teasing the spleen through a 60-μ brass grid using scissors. The cells were washed 3 times in PBS, an resuspended at a concentration of 25×10^6 cells in 100 μl of PBS (f intrathymic injection), or 500 μl of PBS (for intravenous injection Viability of the cells, as determined by trypan blue exclusion, alway exceeded 90%.

Experimental procedure. Under isoflurane inhalation anesthesi the thymus of an intubated male AO rat was exposed after parti median sternotomy. With the aid of an operating microscope (12.5× 25×10^6 PVG splenocytes were injected into the recipient's thymu (50 μl into either lobe) using a 30-gauge needle. Leakage of cells fro the thymus was prevented by compression of the injection site wit a cotton-tipped stick. After the wound was closed, the heart wa heterotopically transplanted into the right side of the neck, using th modified cuff technique of Heron (*9*). The donor aorta and pulmonar

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* Abbreviations: ALS, antilymphocyte serum; CsA, cyclosporin MST, median survival time; STITTI, simultaneous transplantatio and intrathymic tolerance induction.

artery were anastomosed end-to-end to the recipient's carotid artery and jugular vein, respectively. Ischemia was consistently less than 20 min, and all grafts started beating promptly after revascularization. The wound was closed and 1 ml of rabbit ALS (Accurate Chemical Corp., Westbury, NY) was injected intraperitoneally.

In the case of intravenous injection, 25×10^6 splenocytes (in 500 μl of PBS) were injected into the tail vein.

Treatment with CsA (50 mg/ml, Sandoz Pharma AG, Basel, Switzerland) consisted of three 15 mg/kg body weight injections on days 1, 2, and 3.

Graft function was assessed regularly by palpation. Graft rejection, defined as complete cessation of ventricular contraction, was confirmed histologically by staining of the excised heart with R73 (*10*), HIS40 (*11*), HIS48 (*12*), and ED1 (*13*).

RESULTS

The STITTI protocol. Our STITTI protocol consists of an intrathymic inoculation with allogenic splenocytes and injection of ALS intraperitoneally, simultaneously with the transplantation of an allogenic heart on day 0, followed by injections of CsA on days 1, 2, and 3. Of 16 animals thus treated, 14 have accepted their cardiac allografts (Table 1, group 1). This procedure is clearly highly successful, as the survival rate at 150 days after transplantation is over 80%. In a number of cases, the follow-up continued until the natural death of the animal, which proved that acceptance of allografts, in most—if not in all—cases, was permanent.

Relevance of alloantigen and the route of injection. To test whether intrathymic injection with alloantigen was a prerequisite or whether "manipulation of the thymus" would be sufficient, we injected syngeneic instead of allogenic splenocytes into the thymus without changing the remainder of the protocol (group 2). Under these conditions, all 6 animals rejected their cardiac allografts within 28 days after transplantation, which indicates that alloantigen is indeed necessary to induce tolerance.

We also tested wether adding an intravenous inoculation of donor splenocytes to the protocol described above would induce tolerance to cardiac allografts (group 3). Eight animals were treated according to this protocol, of which 6 animals rejected their allografts with a median survival time (MST) of 23 days; the other 2 animals accepted theirs permanently.

Effects of intrathymic inoculation, ALS, and CsA. The median survival time of cardiac allografts in animals treated with intrathymic splenocytes (group 8, MST = 7 days) was not significantly different from that of untreated controls (group 9, MST = 6 days).

Treatment with an intrathymic inoculation of donor splenocytes plus ALS (group 5) or ALS only (group 7) increased the MST of the cardiac allograft to 13 days. As such, the ALS treatment with or without intrathymic inoculation of allosplenocytes did not induce significant prolongation of allograft acceptance.

Injecting the animals with CsA (intramuscularly) on days 1, 2, and 3 did, as expected, prolong the survival of the allograft. However, eventually, the graft was always rejected (group 6, MST = 21 days). Addition of an intrathymic injection with allogenic splenocytes to this protocol increased the MST of the allografts to 26 days, with 1 out of 6 animals permanently accepting the cardiac allograft (group 4).

DISCUSSION

The results presented here clearly indicate that our protocol for STITTI induces acceptance of cardiac allografts in the high-responder PVG to AO rat strain combination without the need for prolonged immunosuppressive treatment (Table 1, group 1). As we now can perform the actual transplantation simultaneously with the beginning of the tolerance induction protocol, this procedure clearly has clinical potential, a feature earlier protocols lacked due to the interval between the tolerance induction and the actual transplantation (*1-4*).

So far, we have treated 16 animals, of which only 2 rejected their allograft, giving us an initial success rate of more than 87%. In addition, long-term studies show that over 80% of the animals still have functioning cardiac allografts 150 days after transplantation.

An explanation for the cessation of function of 2 cardiac allografts on days 25 and 49 might be heart failure due to the lodging of a clot in one of the coronary arteries. However, this could not be confirmed histologically due to the time between cessation of function and autopsy.

Matsuura et al. (*8*) recently described a protocol in which short-term FK506 treatment combined with intrathymic inoculation of allogenic bone marrow cells was successful in

TABLE 1. Results from the STITTI protocol and controls

Group	Treatment[a]				Graft survival (days)
	IT	IV	ALS	CsA	
1	Allo	−	+	+	431[b], 337[b], 315[b], 281[b], 280[c], 211[c], 210[b], 198[c], 185[c], 156[d], 141[c], 119[c], 119[c], 99[b], 25, 49
2	Syn	−	+	+	28, 26, 24, 24, 21, 21
3	Syn	+	+	+	249[d], 179[d], 30, 24, 24, 24, 23, 23
4	Allo	−	−	+	515[b], 28, 28, 28, 26, 26, 26
5	Allo	−	+	−	15, 13, 11
6	Saline	−	−	+	22, 21, 21
7	Saline	−	+	−	14, 13, 13, 6
8	Allo	−	−	−	7, 7, 7
9	Saline	−	−	−	13, 6, 6, 6, 6

[a] Treatment: IT, intrathymic injection with allogenic splenocytes, syngeneic splenocytes, or saline; IV, intravenous injection with allogenic splenocytes; ALS, one 1-ml i.p. injection of ALS; CsA, 3 injections of 15 mg/Kg CsA i.m.

[b] The animal died with a functioning allograft.

[c] The animal was killed with a functioning allograft.

[d] The animal is still alive with a functioning allograft.

obtaining acceptance of simultaneously transplanted allogenic hearts in a BN to Lew rat strain combination. However, no effect of the intrathymic inoculation was demonstrated, as long-term acceptance of the allogenic heart could also be obtained with FK506 only. An effect of the intrathymic inoculation was shown in combination with ALS (>150 days vs. 29.5 days for ALS alone). We are unable to induce acceptance with ALS in combination with allogenic splenocytes intrathymically (group 5), nor does CsA alone induce long-term acceptance of the allograft (group 6). In addition, Prop et al. (*14*) have shown that a single injection of CsA is sufficient to induce acceptance of lung allografts in this combination. From these results, we conclude that the BN to Lewis strain combination used in these experiments does not mount as vigorous a rejection response as the PVG to AO strain combination.

We did not expect intravenous injection of allosplenocytes to induce tolerance; however, 2 out of 8 animals treated with an intravenous inoculation did accept their cardiac allografts (group 3). Two possible explanations for the intravenously induced allograft acceptance come to mind. The mechanisms responsible for intravenous and intrathymic induction of tolerance may be different and the intrathymic mechanism is just the most efficient. Alternatively, intravenous injection of antigen is just an inefficient method of intrathymic tolerance induction. Experiments done by Binder et al. (*15*) suggest that the first possibility is the most likely.

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Prolonged Allogeneic and Xenogeneic Microchimerism in Unmatched Primates without Immunosuppression by Intrathymic Implantation of CD34$^+$ Donor Marrow Cells[1]

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Engraftment of stem cell-enriched donor marrow implanted in the thymus of a foreign host might facilitate acceptance of donor-specific organ or tissue grafts. To test this hypothesis, allogeneic and xenogeneic CD34$^+$ marrow cells from unrelated adult male baboons and humans were injected intrathymically in eight infant female baboons, both with and without standard cyclosporine-based immunosuppression. In allogeneic experiments, male (donor) cells, of both T- and B-cell lineages, were detected by PCR in the peripheral blood of all six recipients and persisted for at least 15 months in 2/4 recipients studied longtutudinally. Donor-derived skin grafts survived twice as long as third party grafts in unimmunosuppressed recipients. In xenogeneic protocols, human male (donor) cells were demonstrable for 7 and 15 months, respectively, in two baboon recipients with evidence that implanted human CD34$^+$ cells had produced lymphoid progeny. Survival of donor-specific skin xenografts was prolonged in one of two recipients. These experiments demonstrate that the intrathymic injection of CD34$^+$ marrow cells can result in long-lasting lymphohematopoeitic microchimerism in unrelated primates even without immunosuppression and can alter donor-specific skin graft survival. © 1997 Academic Press

INTRODUCTION

Despite dramatic improvements in the early graft survival of transplanted solid organs, the long-term survival of transplant recipients remains limited by late graft loss due to chronic rejection and the side effects of chronic immunosuppression: renal failure, infection, and malignancy. All antirejection therapies to date have been based on recipient immunosuppression. An alternative method for prolonging graft survival would be the induction of tolerance, the coexistence of foreign cells or tissues in the host without exogenous immune suppression. A method for inducing recipient tolerance to solid organ grafts might eliminate chronic rejection and allow graft survival without immunosuppression.

Starzl and others (1–4) have observed that human organ transplant recipients who have normal graft function long term on no or minimal immunosuppression often have "microchimerism," defined as the presence of very small numbers of donor-derived cells in blood and other tissues (5). The principle question concerns the extent to which the observed microchimerism is the etiology of the "tolerant" state or simply a result of it.

Tolerance for, or acceptance of, transplanted allogenic tissues was first achieved in rodents by donor marrow transplantation following myeloablation, essentially replacing the recipient lymphohematopoietic cells with those of donor origin (6–9). Although feasible, formal allogeneic bone marrow transplantation after myeloablative therapy is not performed in humans with solid organ transplants because of the associated toxicities, in particular graft versus host disease, as most solid organ transplants are between donors and recipients who are neither related nor fully HLA-matched.

Studies in rodents from several laboratories strongly support the hypothesis that lymphohematopoietic mixed chimerism may be sufficient for the development of a

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tolerant state (10–12). Current efforts to produce mixed chimerism with nonlethal conditioning regimens consisting of lower doses of radiation in conjunction with immunosuppressive drugs have resulted in acceptance of skin and cardiac grafts in rodents (13, 14) and prolonged survival of renal allografts (15) and xenografts (16) in nonhuman primates. Yet it remains to be determined if stable lymphohematopoietic mixed chimerism can be achieved in a clinically applicable protocol.

In nonhuman primates, the infusion of unfractionated donor marrow or donor blood and marrow cells depleted of $CD3^+$ and/or $HLA\text{-}DR^{+bright}$ cells along with anti-thymocyte globulin (ATG) has produced transient microchimerism and prolonged the survival of renal allografts (17, 18). In clinical trials using infusions of unfractionated donor marrow or blood in renal transplant recipients, reduced rejection rates have been reported for recipients who developed microchimerism (19) or suppressed MLR to donor cells (20). The increased rejection rates among recipients who did not become chimeric may have been due to sensitization by the cell infusions. Implantation of the donor marrow fractions directly into the thymus might provide a protected environment in which donor cells could proliferate without inducing allosensitization and provide a source of donor-derived antigens for T-cell selection during development.

Maintenance of the tolerant state appears to require the continued presence of donor antigens (18, 21–23). In rodent models, the intrathymic implantation of donor marrow, spleen, or tissue cells, as well as histocompatibility antigens, with or without immunosuppression, has led to acceptance of allogenic organ and tissue grafts (24–30). Since mature hematopoietic and somatic cells and peptide antigens all may have a finite lifespan, even in the thymus (31), tolerance either becomes dependent on chimerism, whether hematopoietic (18, 21) or intrathymic (22, 23), or becomes dependent on the presence of the organ graft (15, 30, 32, 33). The development of persistent hematopoietic microchimerism has not been reported to date in intrathymic injection models, nor in infusion models without myelosuppressive conditioning (34, 35). The implantation of hematopoietic stem cells, the source of all mature lymphohematopoietic cells, would provide, in theory, a self-renewing source of donor antigens for maintenance of the tolerant state. Marrow repopulating stem cells were first shown to express the CD34 antigen in baboons (36), and subsequently in humans and mice (37, 38). Purified $CD34^+$ marrow cells can completely repopulate the lymphohematopoietic system *in vivo* (39).

The critical question is whether purified hematopoietic stem cells injected into the thymus or another hematopoietic organ can engraft in the absence of myeloablation and, if so, if the resulting chimeric state alters immunologic recognition of donor tissues. In the present studies, we have used baboons as a model in which to investigate whether purified allogeneic and xenogeneic, human, $CD34^+$ marrow cells injected directly into the thymus will engraft in the absence of immunosuppression and myeloablative therapy. We then examined whether this influenced the survival of donor-specific skin grafts.

MATERIALS AND METHODS

Animal Care

Young juvenile female recipient (8 to 12 weeks of age) and male third party baboons (*Papio cynocephalus anubis*) used in this study were obtained from the University of Washington Regional Primate Research Center's (RPRC) breeding facility and cared for at the RPRC Infant Primate Center. An adult (3-year-old) male baboon, which served as the allogenic marrow donor, was obtained from the Columbia University (New York) primate colony to ensure unrelatedness. Studies were conducted under Animal Care and Use Committee-approved protocols and under American Association for Accreditation of Laboratory Animal Care-approved conditions. All minor procedures, including blood draws, bone marrow aspirations, skin grafts, and skin graft biopsies, were performed after animals had been anesthetized with a combination of ketamine-HCl (Aveco, Fort Dodge, IA) and xylazine (Haver, Shawnee, KS). Intrathoracic marrow cell implant procedures were performed under general anesthesia, using endotracheal intubation and ventilation with isofluorane in 100% oxygen.

Isolation and Intrathymic Injection of $CD34^+$ Marrow Cells

Marrow was obtained from adult male baboons by aspiration of both femurs and humeri under general anesthesia as previously described (36). Human cadaveric marrow and skin were obtained from the Northwest Tissue Center (Seattle, WA) from adult male donors (blood groups O and A) whose families consented to tissue donation for research purposes. Human cadaveric marrow was isolated from vertebral bodies (40), and split thickness human donor skin was cryopreserved from the same donors.

$CD34^+$ marrow cells not expressing T- and B-cell antigens were isolated from baboon and human marrow by using the anti-CD34 antibody 12.8 (41) with two-color fluorescence-activated cell sorting (FACS) as previously described (39). Briefly, baboon $CD34^+$ marrow cells for intrathymic injection were first enriched by positive selection on an avidin–biotin immunoadsorption column (Ceprate, CellPro, Bothell, WA) using biotin–conjugated 12.8 (anti-CD34, mouse monoclonal IgM). The enriched $CD34^+$ cells were then labeled again with unconjugated 12.8 and with mouse monoclonal IgG antibodies 9.6(CD2), G17.2(CD4), 51.1(CD8), S8S(CD10), 1F5 or B1(both CD20), and 5B12 (a 40-kDa antigen on baboon neutrophils), and then stained with

phycoerythrin (PE)-conjugated anti-mouse IgM antiserum (Calbiochem, La Jolla, CA, or Biomeda, Foster City, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG specific antiserum (Kirkegaarde and Perry, Gaithersburg, MD) for FACS selection of CD34-positive, lineage-negative cells. Baboon $CD34^+$ marrow cells for PCR analysis were prepared by negative selection using immunomagnetic beads (Dynal, Oslo, Norway) to deplete marrow cells expressing lineage-associated antigens identified by the above mouse monoclonal IgG antibodies and antibodies G28.5-(CD28) and 5A9 (to a baboon CD-11-like determinant), with subsequent sorting for $CD34^+$, lineage negative cells as above. For the human marrow donors, $CD34^+$ cell injectates were enriched from cryopreserved human cadaveric marrow first by negative selection using immunomagnetic beads to deplete cells expressing lineage antigens identified by the IgG monoclonal antibodies 35.1(CD2), 24.1(CD10), 60.1(CD11b), HD37-(CD19), 1F5(CD20), p67.6(CD33), F13(CD36), R10(glycophorin A), and 7B9 (42). These enriched cells were then labeled with 12.8 and relabeled with the antibodies used for depletion and then stained with the anti-IgM-PE and anti-IgG-FITC antisera for FACS.

As controls, unseparated marrow buffy coat cells were labeled with the IgM antibody H12C12 (anti-mouse Thy 1.2) and the IgG antibodies 31.A and 1A14 (anti-mouse Thy 1.1) and then stained with the anti-IgM specific and anti-IgG specific antisera. Human and baboon $CD34^+$ cells that did not bind the antibodies used for negative selection were separated by two-color FACS as previously described (39). Analysis of the sorted baboon and human $CD34^+$ marrow cells showed that they were >98% $CD34^+$ and devoid of detectable mature T and B lymphocytes and myeloid cells.

Purified $CD34^+$ marrow cells were injected into the thymus of eight female recipient baboons via a left anterior thoracotomy. Six animals received 1×10^6 purified $CD34^+$ (>98% $CD34^+$) marrow cells from unrelated male baboons, divided into five equal amounts and injected directly into five sites in the left lobe of the thymus. Two animals received 6×10^5 purified $CD34^+$ marrow cells from a human male donor (blood group O), injected into both lobes of the recipient thymus. Peripheral blood samples were taken at intervals to assess the presence and persistence of donor cells using polymerase chain reaction (PCR) to amplify baboon or human sequences specific for the Y chromosome (43, 44).

Immunosuppression

Two animals given allogeneic baboon cells and one animal given xenogeneic human cells were placed on triple drug immunosuppression to simulate the regimen used in clinical cardiac transplantation. Immunosuppression was begun on the day of donor cell implantation and continued for 6 months. Cyclosporin A (Sandoz Pharmaceuticals, East Hannover, NJ) was loaded using twice daily (b.i.d.) subcutaneous injections of 12 mg/kg/day until the desired level was reached, 500–800 ng/ml, measured by high power liquid chromatography in whole blood. The drug dose was then tapered, maintaining levels, to an average maintenance daily dose of 4 mg/kg b.i.d. Azathioprine was given at dose of 2 mg/kg/day in addition to dexamethasone, beginning with an initial dose of 2.5 mg/kg twice daily, administered intramuscularly, tapering to 1.0 mg/kg b.i.d.by the end of the first week, 0.5 mg/kg b.i.d. by the end of the first month, and down to a maintenance dose of 0.25 mg/kg b.i.d. after 2–3 months. No myeloablative therapy or anti-lymphocyte serum induction therapy was employed.

Four animals given allogeneic and one animal given human $CD34^+$ marrow cells did not receive any immunosuppression.

Skin Grafts from Allogenic and Xenogenic Donors

Full thickness skin allografts were excised from the back of a donor baboon with a round punch bioptome, either 6 or 13 mm in diameter. A matching defect was created in the dorsal skin of the recipient animal and the graft inserted, tacking down the graft margins with sutures. Human skin xenografts, 25 × 30 mm, were placed similarly, using split-thickness cryopreserved grafts from the human cadaver donors. Because of the consistency of baboon skin, it was not possible to prepare an equally thin split thickness baboon skin graft, so a full thickness graft of equivalent size from a third party baboon was used as the allograft control for the skin xenografts. Grafts were examined every other day for viability and biopsies taken with a 1-mm punch bioptome. Skin graft recipient animals were preaccustomed to wearing a zippered jacket specially designed to prevent dislodgement of the grafts. By using this jacket, neither restraints nor plaster casts were required to hold skin grafts in place.

The time to graft rejection was defined as the number of days until the graft became completely necrotic, both dry and black in coloration. Necrosis was usually observed several days earlier than the actual sloughing of the necrotic graft eschar from the bed.

Skin grafts biopsies and excised specimens were parafin-embedded, fixed in methyl Carnoys solution, and stained with hematoxylin and eosin for histology. Serial sections were frozen in Tissue Tek OCT compound for standard immunocytochemistry using antibodies to CD3 and CD68 (both from Dako Corporation, Carpinteria, CA), detected by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), and visualized by the ABC Elite avidin–biotin peroxidase technique.

Polymerase Chain Reaction (PCR) for Baboon and Human Y Chromosome-Specific Sequences

PCR was used to identify a 174-basepair (bp) sequence specific to male baboons using primers de-

scribed by Reitsma *et al.* (43) or a 108-bp DYZ-1 sequence specific to human males using primers described by Lo *et al.* (44). Unfractionated peripheral blood lymphocytes (PBL) or bone marrow buffy coat cells, free of red cells, and subpopulations of PBL and bone marrow cells isolated by two cycles of FACS, were used for PCR. Replicate samples of viable cells (10^5 cells per sample) were prepared for PCR after the methods of Reitsma *et al.* (43). Briefly, 40 cycles of amplification were done, with cycles 1 and 2 using denaturation at 94°C for 2 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min, and cycles 3–40 using denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and amplification at 72°C for 1 min. PCR products were analyzed by ethidium bromide agarose gel (4%) electrophoresis. The detection of human male sequences in the xenograft experiments was also performed independently by a second laboratory.

For the allograft experiments, the controls for each amplification included (i) a sample of water only, without DNA, (ii) a sample of 10^5 cells from a normal female baboon, (iii) 10^5 cells from a normal male baboon, and (iv) in some instances, 10^5 cells from dilutions of normal male baboon cells in normal female baboon cells. For the xenograft experiments, the same controls were used, substituting human male and female cells for baboon cells. Additional controls using cells from normal male and female baboons showed that no product was amplified using primers for the human DYZ-1 sequence (see controls in Fig. 3B). To determine the sensitivity of the PCR reactions, we analyzed varying dilutions of normal male baboon and human PBL in PBL from normal female baboons or humans. With either PCR technique, we could detect as few as 10 to 100 (0.01 to 0.001%) male cells in 100,000 female cells.

PCR for Baboon Major Histocompatibility Complex (MHC) Sequences

PCR was performed for regions homologous to the second exon of human HLA-DRβ, DQα, and DQβ genes as described (45). Hybridization with sequence-specific oligonucleotide probes was used to define unique and shared sequence motifs between donors and recipients. A total of 109 probes were used in this analysis, the majority based on sequences from the second hypervariable region. The mismatching of selected pairs was intended to provide primers for donor-specific amplification. Pairs were mismatched at one to three loci, the equivalents of DRβ, DQβ, or DQα.

Each marrow cell recipient was mismatched for multiple sequence motifs (6 to 9) with the marrow cell donor. The third party skin graft donors were selected to have at least one unique sequence motif not present in the skin graft recipient nor in the marrow cell donor.

Detection of Alloantibody

To detect the development of anti-donor IgG antibodies in both allograft and xenograft recipients, recipient plasma samples were obtained following marrow cell implantation and skin grafting. FITC-labeled goat antibodies to human Fcγ and Fcμ (Jackson ImmunoResearch Laboratories, West Grove, PA), which cross-react with baboon, as well as human, IgG and IgM, were used to detect antibodies binding to donor, third party, and autologous cells. The presence of alloantibody was determined using a two-color immunofluorescence technique and analyzed on a flow cytometer (Becton–Dickinson Flow Cytometry Systems, San Jose, CA). A channel shift of greater than 30 channels compared to control was considered indicative of antibody binding. Only antibody in recipient serum which bound to donor but not to autologous cells was interpreted as antibody to donor alloantigen or xenoantigen.

RESULTS

Intrathymic Implantation of Allogeneic CD34$^+$ Marrow Cells Produces Hematopoietic Microchimerism with or without Immunosuppression

Purified allogeneic CD34$^+$ marrow cells from an unrelated male baboon were injected into the left thymic lobe of each 8- to 12-week-old female recipient. Two recipients were given immunosuppression and four received no immunosuppression. Using PCR to detect a male-specific sequence (Fig. 1), we were able to detect male cells in samples of PBL from all six female recipients. In four marrow cell recipients without later skin grafts (i.e., no other source of donor antigens), male donor cells were detected as early as 1 week after injection in three animals, and remained detectable for 6–7 months in three animals and up to 15 months in the two immunosuppressed animals (Table 1). The frequency of male cells in the marrow and blood of recipient animals was unlikely to have exceeded 0.1% (1 in 1000 cells). Although formal limiting dilutional analysis of samples was not performed, since the limit of

FIG. 1. Allogeneic chimerism. Representative longtitudinal PCR analysis of peripheral blood and marrow cells from two female baboons after intrathymic injection of purified baboon male CD34$^+$ marrow cells. Ethidium bromide-stained gel showing 174-bp PCR reaction product specific for a male (Y chromosome) determinant. Lane 1, water, no DNA; Lane 2, 10^5 PBL from normal female baboon; Lane 3, 10^5 PBL from female baboon F93011 1 week after donor marrow cell injection; Lane 4, 3 weeks after injection; Lane 5, 4 weeks after injection; Lane 6, 5 weeks after injection; Lane 7, 6 weeks after injection; Lane 8, 7 weeks after injection; Lane 9, 10^5 marrow cells from female baboon M92523 19 weeks after injection; Lane 10, 10^5 PBL from normal male baboon.

TABLE 1

Analysis of Blood and Marrow for Donor (Male) Cells from Four Infant Female Baboons Following Direct Intrathymic Injection of Purified Allogeneic CD34+ Marrow Cells from an Unrelated (Male) Donor

Sample	Weeks post cell injection	No. samples with positive PCR/No. samples tested			
		Not immunosuppressed		Immunosuppressed	
		M92522	F93011	F92520	M92523
PBL	1	3/3	2/3	0/3	1/3
PBL	2	2/3	ND	1/3	1/3
PBL	3	3/4	3/4	ND	ND
PBL	4	ND	3/3	1/3	2/4
PBL	5	3/4	3/4	2/4	3/4
PBL	6	0/3	2/3	0/3	2/4
PBL	7	3/4	3/3	0/3	0/3
PBL	8	1/2	1/2	0/3	2/3
PBL	9	4/4	0/2	0/3	1/3
PBL	10	1/2	ND	0/3	1/3
PBL	11	1/3	ND	ND	ND
PBL	17–19	1/3	ND	1/3	3/3
PBL	20–22	0/2	1/3	1/2	1/2
PBL	23–29	1/6	2/5	2/9	2/4 [7×10^4][a]
PBL	46–48	0/1	ND	0/1	0/1
PBL	60–62	0/1	0/1	1/1	1/1
CD2+ PBL	20–22	0/2	1/2	2/3 [6×10^4][a]	2/2
CD2+ PBL	26–28	0/2	1/1 [4×10^4][a]	4/5 [1×10^4][a]	1/2 [7×10^3][a]
CD20+ PBL	20–22	0/2	0/1	2/2 [5×10^3][a]	1/2
CD20+ PBL	26–28	0/2	0/1 [5×10^3][a]	0/5	1/2 [10^3][a]
BM	17–19	0/2	ND	0/3	2/3
CD34+ BM	27–29	0/1	0/2	0/2	1/2 [10^4][a]
BM	46–48	1/1	ND	ND	ND

Note. Replicate samples of unseparated PBL and BM (10^5/sample) and double-sorted CD2+ (T cells), CD20+ (B cells) from blood, and CD34+ cells from marrow were assayed by PCR for the presence of a unique male-specific 174-bp sequence (43). ND, not done.

[a] Numbers in brackets represent the number of cells in each sample tested if different from 10^5.

detection was 0.01% male cells (see Materials and Methods) and not all replicate samples tested were positive at any given time point, these findings are consistent with a very low level of chimerism.

To determine if the male, or donor, cells present in the circulation represented hematopoietic cells, we used flow cytometry to purify CD2+ T cells, CD20+ B cells, and CD34+ cells from the peripheral blood and marrow of four animals between 20 and 30 weeks after intrathymic injections. Skin grafts were not performed in these four recipients, so the intrathymic injections were the only source of donor cells. Male (donor) cells were detected by PCR in CD2+ T cells and CD20+ B cells from blood as well as in the CD34+ cells from marrow. Since donor T and B cells were depleted prior to intrathymic implantation, this finding suggests that the injected donor CD34+ cells had given rise to progeny at least in the T- and B-lymphoid lineages.

Intrathymic CD34+ Marrow Cell Recipients Have Prolonged Survival of Donor-Specific Skin Allografts

Given that hematopoietic microchimerism was observed after intrathymic injection of allogeneic CD34+ marrow cells, we investigated whether this resulted in altered recognition of donor-derived skin grafts compared to skin grafts from self (autologous) or from another unrelated baboon that had not served as the marrow cell donor (third party).

Two recipient animals each had two sets of skin grafts placed 6 and 14 weeks after intrathymic injection of CD34+ marrow cells (Table 2). Neither skin allograft recipient received immunosuppression, at the time of either marrow cell implantation or skin grafting. For the first set of grafts at 6 weeks, nine 6-mm-diameter grafts were placed on each recipient animal: three from autologous skin, three from the marrow cell donor, and three from an unrelated third party baboon. Skin allografts from the marrow cell donors survived 42 days in both marrow cell recipients, markedly longer than third party allografts, all of which appeared necrotic by day 10, with the graft eshar sloughing by day 24 (Fig. 2). Autologous skin grafts survived indefinitely. In one recipient, a biopsy of the skin graft from the marrow cell donor on day 27, when it still appeared viable and 3 days after complete loss of the third party allograft, confirmed the presence of male donor cells by PCR.

TABLE 2

Skin Graft Survival (Days to Necrosis) in Allogeneic and Xenogeneic Marrow Cell Recipient Baboons and in Control Baboons without Marrow Cell Implants

Skin graft type	Skin graft donor:	Marrow cell recipients: Marrow cell donor	Marrow cell recipients: Third party donor	Control recipients
Allogeneic				
No immunosuppression				
First set		42, 42, 42, 42, 42, 42[a]	10, 10, 10, 10, 10, 10[a]	10, 10, 10, 10, 14 (fresh) 10, 10, 10 (cryopreserved)
Second set		17, 17, 21, 21[b]	13, 13, 13, 13[b]	
Xenogeneic				
No immunosuppression				
First set		28[c]	10 (baboon)	10, 10, 10[c]
Second set		11	11 (baboon); 11 (human)[c]	
With immunosuppression				
First set		15[c]	18 (baboon)	
Second set		11	11 (baboon); 11 (human)[c]	

Note. Allogeneic skin grafts were from unmatched baboon donors. Xenogeneic skin grafts were from human male donors.
[a] Three allogeneic skin grafts were placed on each recipient animal from each donor.
[b] Two allogeneic skin grafts were placed on each animal from each donor.
[c] All human grafts are cryopreserved skin.

Gross examination of the first set of skin allografts showed an indurated margin around the periphery of the graft from the marrow cell donor which was not seen either in the autografts or in the third party allografts (Fig. 2). This phenomenon occurred in both recipients.

The second set of skin allografts was then placed on each skin graft recipient animal, consisting of four larger, 13-mm-diameter grafts: two from the marrow cell donor and two from the same third party baboon. In the second set allografts, the necrotic process appeared to begin earlier, with all grafts having some dark discoloration by day 7. Third party allografts were frankly necrotic by day 13, whereas the grafts from the marrow cell donors took until days 17 and 21, respectively, to develop full necrosis.

Intrathymic CD34$^+$ Marrow Cell Implants Do Not Prevent the Development of IgG Alloantibodies to the Marrow Donor

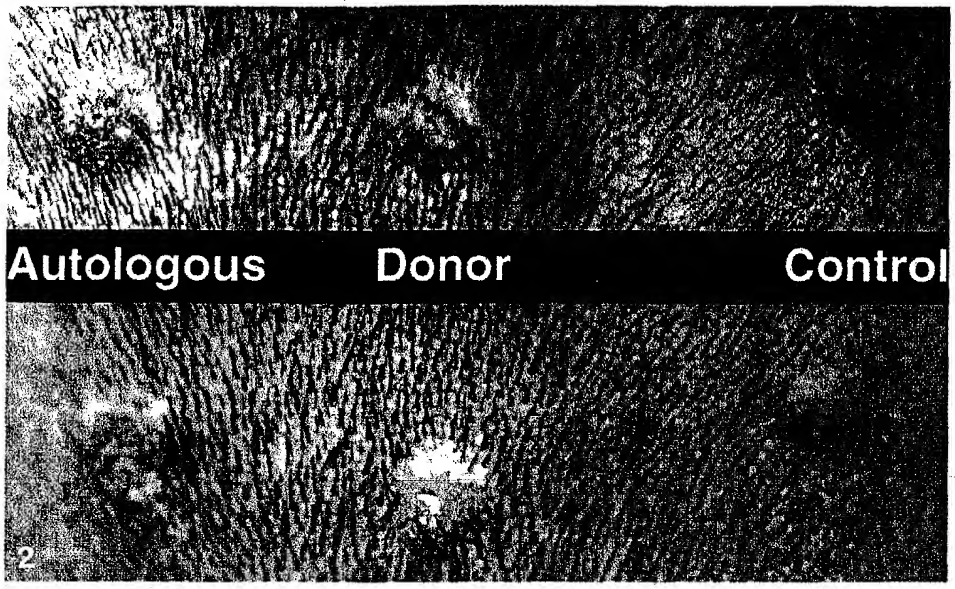
Neither of the two recipients of allogeneic marrow cells had detectable IgG alloantibodies following donor CD34$^+$ cell intrathymic injections prior to skin grafting. However, both developed IgG alloantibodies to the marrow cell donor by 10 days following the first set of skin grafts. An IgM response was not detected (Table 3). Two age-matched control animals, without marrow cell injections, demonstrated both IgG and IgM responses to skin grafts from allogenic baboon donors. One of the two control baboons had preformed IgG alloantibodies to T and B cells from a random donor, which probably represented residual maternal antibodies.

Intrathymic Injection of Purified Human CD34$^+$ Marrow Cells Produces Prolonged Xenogenic Microchimerism in Baboon Hosts

Purified CD34$^+$ human marrow cells from a male cadaveric donor were injected intrathymically into two juvenile female baboons, one immunosuppressed and one not immunosuppressed. PBL samples, analyzed by PCR for the 108-bp DYZ-1 sequence (44), demonstrated the presence of low levels of human male cells in the circulation as early as 2 weeks after injection in one animal. To determine that the human male cells present in blood represented hematopoietic cells, CD34$^+$ as well as CD3$^+$ cells, enriched from peripheral blood, were assayed by PCR in each recipient animal. Evidence of human male (donor) cells in both CD3$^+$ and CD34$^+$ fractions was found, supporting the hematopoietic potential of the human male cells in the blood of these baboons (Fig. 3).

Human cells were still detectable for over 7 months postimplant in the nonimmunosuppressed baboon and identifiable at 15 months in the immunosuppressed recipient. PBLs were sampled from both animals on 15 occasions during the first 8 months following intrathymic injection (Table 4). One animal, interestingly the one without immunosuppression, had donor cells detectable on 13 of these samplings.

PCR analysis of dilutions of normal human male PBL in human female PBL demonstrated that the limit of detection of the human male cells in this assay was at 1:5000. Since not all replicate samples were positive by PCR, this suggests that the frequency of human male cells in the blood of recipients baboons was at least 1 in 5000 cells.



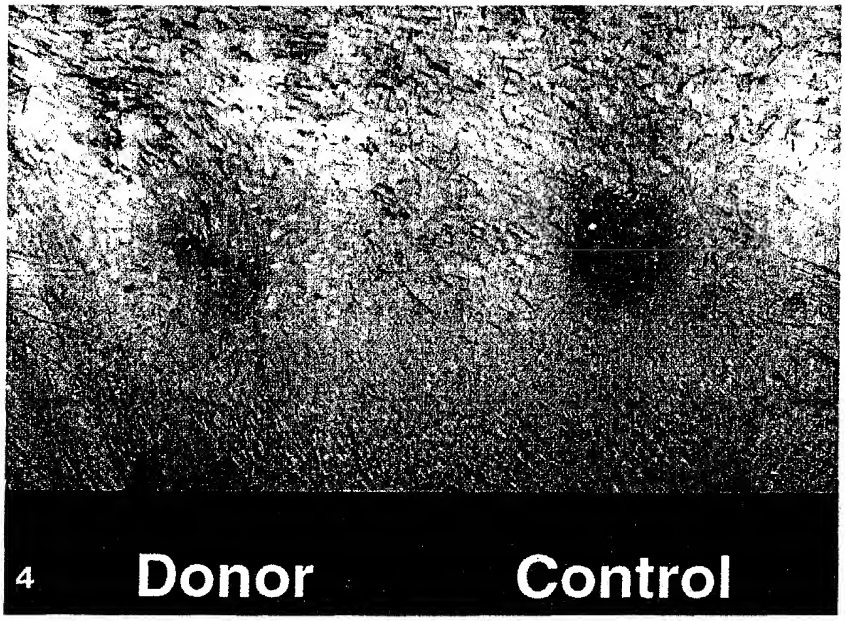


FIG. 2. Skin allografts, 27 days. First set skin grafts 27 days after grafting onto a female baboon who underwent intrathymic injection of purified allogeneic male baboon CD34+ marrow cells 6 weeks previously. On the left, autologous skin grafts which are fully incorporated. In the center, skin allografts from the male baboon that donated the CD34+ marrow cells. At 27 days, the grafts from the marrow cell donor are still pink and viable. The raised margins of these grafts are visible, compared to the autologous grafts. On the right, skin allografts from an unrelated (third party) female baboon which became necrotic after 10 days—only a black eschar remains at the graft site.

FIG. 4. Skin xenografts, 22 days. First set skin grafts from the human CD34+ marrow cell donor and from a third party unrelated baboon at 22 days following primary skin grafting. On the left is the skin graft from the human marrow cell donor ("Donor"), which still appears viable. A small red spot is the site of a previous 1-mm biopsy, submitted for PCR, which confirmed the persistence of donor DNA in this graft. On the right is the control allograft ("Control") from an unrelated third party baboon which is necrotic.

Skin Xenograft Survival Was Prolonged in the Nonimmunosuppressed Recipient Baboon with Persistent Chimerism

First and second set skin xenografts were placed on the two baboon recipients of human CD34+ marrow cells. Thirteen weeks following the marrow cell injections, 13-mm-diameter skin grafts, one from the human marrow donor (blood group O) and one from a third party baboon, were placed on each of the two xenogeneic marrow cell recipients. In the xenograft

TABLE 3

Antibody Binding to T Lymphocytes Obtained from Marrow Cell and Skin Graft Donors

Baboon	Serum drawn (POD)[a]	Flow crossmatch (channels > negative control)[b]			
		Donor 1		Donor 2	
		IgG	IgM	IgG	IgM
Allogeneic marrow cell recipients and controls challenged with allogeneic skin grafts					
Marrow cell recipient 1[c]	10	POS (36)	NEG (0)		
	21	POS (95)	NEG (0)		
	28	POS (89)	NEG (0)		
Marrow cell recipient 2	10	POS (120)	NEG (0)		
	21	POS (125)	NEG (0)		
	28	POS (104)	NEG (0)		
Control skin graft recipient A	0	NEG (2)	NEG (0)	POS (36)	NEG (4)
	7	NEG (15)	NEG (14)	POS (54)	NEG (14)
	17	POS (40)	POS (69)	POS (89)	POS (66)
	36	POS (83)	NEG (15)	POS (117)	NEG (8)
Control skin graft recipient B	0	NEG (0)	NEG (19)	NEG (0)	NEG (27)
	7	NEG (0)	NEG (11)	NEG (9)	NEG (15)
	22	POS (36)	POS (41)	POS (71)	POS (39)
	41	POS (51)	NEG (18)	POS (69)	NEG (20)

Baboon	Serum drawn (POD)[a]	Flow crossmatch (channels > negative control)[b]	
		IgG	IgM
Xenogeneic marrow cell recipients and controls challenged with xenogeneic skin grafts			
Human marrow cell recipient (immunosuppressed)	10	NEG (18)	POS (46)
	28	POS (112)	POS (53)
	60	POS (79)	POS (36)
Human marrow cell recipient (not immunosuppressed)	10	POS (132)	POS (101)
	28	POS (147)	POS (54)
	60	POS (143)	NEG (27)
Control skin graft recipient A	0	NEG (29)	POS (70)
	7	POS (39)	POS (37)
	17	POS (114)	POS (97)
	36	POS (149)	POS (106)
Control skin graft recipient B	0	NEG (30)	NT[d]
	7	POS (44)	NT
	22	POS (117)	NT
	41	POS (110)	NT

[a] POD means postoperative day: days after placing the primary skin graft.

[b] The flow crossmatches were analyzed on a 4-decade logarithmic scale divided into 256 channels. The channel shift given indicates the increased fluorescence above the same cells incubated with the negative control. A shift greater than 30 channels was considered a significant increase in antibody; an increase of 60 channels indicates a 10-fold increase in the amount of antibody bound to the cells.

[c] Neither the allogeneic marrow cell recipients who received skin grafts nor the control baboons who received skin grafts without marrow cell implants were immunosuppressed. Of the xenogeneic marrow cell recipients, one was and one was not immunosuppressed.

[d] NT, not tested.

protocols, one baboon recipient was given no immunosuppression and one was immunosuppressed from the time of donor marrow cell implantation until after rejection of the second set of skin grafts, 8 months later.

Rejection of the graft from the marrow cell donor occurred by slow contraction rather than frank necrosis (Fig. 4). In the recipient without immunosuppression, the graft from the human marrow cell donor appeared viable out to 28 days, after which the site began to contract slowly (Table 2). PCR analysis of a biopsy of the xenograft skin from the marrow cell donor at 10 and 18 days confirmed the persistence of human donor DNA in the xenograft (Table 4). In comparison, the third party baboon skin allograft became black and necrotic at 10 days.

In the immunosuppressed baboon, the graft epider-

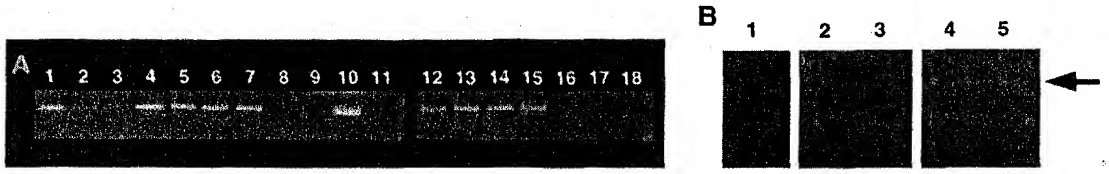


FIG. 3. Xenogeneic chimerism. (A) Representative PCR analysis of peripheral blood cells from two female baboons 33 weeks after intrathymic injection of purified human male CD34⁺ marrow cells. Ethidium bromide-stained gel showing 108-bp DYZ-1 PCR product specific for human male DNA in unsorted, CD34⁺, and CD3⁺ populations. Immunosuppressed baboon: Lane 1, whole blood; Lane 3, Ficoll–Hypaque-separated PBL; Lane 5, CD3⁺-enriched PBL. Baboon without immunosuppression: Lane 2, whole blood; Lane 4, Ficoll–Hypaque-separated PBL; Lane 6, CD3⁺-enriched PBL; Lane 7, CD34⁺-enriched PBL. Controls: Lane 10, 100% human male cells; Lane 11, water, no DNA; Lanes 12 and 13, human male PBL diluted in female PBL, 1:50; Lanes 14 and 15, 1:500; Lanes 8, 16, and 17, 1:5000; Lane 9, 1:10,000; Lane 18, 1:50,000. (B) Species-specific controls. Primers for the human DYZ-1 product do not amplify a product from DNA from normal male and female baboons. Each lane represents PCR on DNA extracted from 10^5 cells. Lane 1, female baboon PBL; Lane 2, male baboon PBL; Lane 3, water (no DNA) control; Lane 4, 10^3 human male PBL in 10^5 female baboon PBL; Lane 5, 10^2 human male PBL in 10^5 female baboon PBL. The arrow points to the 108-bp DYZ-1 PCR product. (A nonspecific band of lower molecular weight is present below in all lanes.)

mis from the xenograft marrow donor was lost on day 15; the dermal bed then slowly contracted over the next 2 weeks. The third party baboon allograft necrosed on day 18. PCR done on graft biopsies on day 10 revealed human donor DNA to be present in the skin xenograft tissue.

Twenty-three weeks following the marrow cell implants, and 7 weeks after rejection of the first set of skin xenografts, a second set of three larger (25 × 30 mm) skin grafts was placed on each xenogeneic cell recipient, using skin grafts from the human marrow donor, from a third party male human cadaver donor (also blood group O), and from a third party male baboon. In the nonimmunosuppressed recipient, evidence of necrosis began in all grafts by day 4 and was complete by day 11. In the immunosuppressed recipent, the grafts from the marrow cell donor as well as both third party xenograft and allograft had visibly necrosed by day 11. Thus, in the second set xenografts, there was no obvious sparing of the graft from the marrow cell donor, nor was accelerated rejection seen.

In spite of skin graft rejection, chimerism persisted in both recipient animals following loss of the second set of skin grafts: chimerism remained detectable for at least 6 more weeks in the nonimmunosuppressed recipient and for over 9 months in the immunosuppressed recipient.

The Development of Xenoantibodies Does Not Preclude Persistence of Xenogenic Chimerism

Preformed anti-human IgG antibodies were not found in five age-matched juvenile control baboons on random

TABLE 4

Evidence for Xenogeneic Chimerism: Analysis of Peripheral Blood Samples and Skin Graft Biopsies for Human Male (Donor) Cells in Two Baboon Recipients of Human CD34⁺ Marrow Fractions

		No. of samples with positive PCR results/ No. of samples					
		Recipient without immunosuppression		Recipient with immunosuppression		Xenograft controls–ratios of human male:female cells	
Timeline	Sample date (weeks post CD34⁺ cell implants)	PBL	Skin	PBL	Skin		
Cell injection	0					1:0	+
	2	1/2		0/2		1:50	+
	7–12	2/8		1/8		1:500	+
Skin graft 1	13	1/2		1/2		1:5000	+/–
	14	1/2		0/2		1:10,000	–
	15	1/2	2/2	2/2	2/2	1:50,000	–
	16	1/2	1/2	0/2	0/2	Water	–
Skin graft 2	23	2/2		0/2		Human F	–
	27	2/2		0/2		Baboon M	–
	28–33	4/8		2/8		Baboon F	–
	62	0/2		1/2			

Note. Replicate samples of unseparated PBL (10^5 cells/sample) were assayed by PCR for the presence of human male cells using a sequence unique to the human, but not baboon, Y chromosome (44). Control samples with serial dilutions of human male in human female cells and normal baboon male and female cells are demonstrated. M, male; F, female.

sampling (data not shown). In contrast, the baboon receiving human marrow cells without immunosuppression demonstrated anti-human IgG antibodies at 10 days following the initial skin grafts (Table 3). In the immunosuppressed baboon, anti-human IgG antibodies were not detected until 1 month following placement of the skin graft from the human marrow donor. In both animals, hematopoietic chimerism persisted beyond the time of xenoantibody appearance, for another 4 months in the nonimmunosuppressed animal, and for 11 months in the immunosuppressed recipient.

Controls for Skin Allografts and Xenografts

To ascertain the persistence of fresh and cryopreserved allograft and xenograft skin grafts in control baboons without stem cell implants, two age-matched juvenile female baboons received 13-mm-diameter skin grafts from various donors without prior marrow cell implantation nor immunosuppression. Grafts consisted of fresh autologous skin ($n = 1$), fresh skin allografts from unrelated female ($n = 3$) and male ($n = 2$) baboons, cryopreserved skin allografts from unrelated male baboons ($n = 3$), and cryopreserved, split-thickness skin xenografts from human male donors ($n = 4$). One of the human skin graft donors was blood group O, the same blood group as the human marrow cell donor in the xenograft experiments, and the other was blood group A.

In these control animals, four of five fresh baboon skin allografts became necrotic by 10 days; the fifth was dark and dusky by day 10 and frankly necrotic at 14 days. The three cryopreserved baboon skin allografts all appeared necrotic by day 10—cryopreservation did not extend skin graft survival. The gender of the allograft donor baboon also did not affect graft survival. The four cryopreserved human skin grafts all became dark brown in color by day 10, and dry and shriveled by day 14 with the graft eschar sloughing off by day 17. Human blood group type did not appear to affect skin graft survival. Skin graft survival on immunosuppressed control baboons was not pursued since only one of the four skin-grafted marrow cell recipients was immunosuppressed (a xenograft recipient) and since third party skin graft survival time in this animal was only slightly prolonged.

DISCUSSION

The current study is the first demonstration that purified allogeneic or xenogeneic $CD34^+$ marrow cells can produce hematopoietic microchimerism in infant primates following intrathymic injection in either the absence or the presence of immunosuppression. Hematopoietic chimerism was detectable in each of the eight baboons after intrathymic injection of unmatched allogeneic or xenogeneic $CD34^+$ marrow cells, whether the left lobe alone or both lobes of the thymus were injected. Five of these eight recipients did not receive any immunosuppression. To mimic clinical solid organ transplantation, adult, rather than fetal, marrow was used as the source of cells and immunosuppressed baboon recipients received standard clinical triple-drug immunosuppression.

Because donors and recipients are unmatched in clinical thoracic transplantation, a baboon marrow cell donor from a different colony was used to ensure unrelatedness. This donor was demonstrated to be mismatched with the recipients in at least multiple sequence motifs corresponding to human HLA-DR and HLA-DQ loci using available molecular probes.

Importantly, our findings suggest that, following intrathymic injection, the allogeneic and xenogeneic $CD34^+$ marrow cells proliferated to produce both lymphoid progeny and cells maintaining a $CD34^+$ phenotype. Since the donor marrow inoculum was T- and B-cell-depleted, we presume that recovered donor T and B cells were derived from the introduced donor stem cells. Also, with all recipients followed for 15 months postimplant, none of the recipients had overt evidence of graft versus host disease, an important determinant of potential clinical applicability.

Achieving long-lasting lymphohematopoietic microchimerism across a xenograft barrier without immunosuppression was unexpected. Certainly, the young age of the baboon recipients and lack of preformed anti-human IgG xenoantibodies may have contributed to the success of these experiments. Other investigators have also described an absence of preformed anti-human xenoantibodies in infant baboons (46). In this study, chimerism persisted from several months to almost a year after the development of xenoantibodies. Persistence of xenogeneic lymphoid chimerism following first set skin graft rejection, as seen in this study, has been reported in mixed xenogeneic chimeras in irradiated rodents (12); here, low levels of chimerism persisted even after the rejection of second set grafts.

Lymphohematopoietic microchimerism was also produced with a relatively low dose of cells (6×10^5–1×10^6 cells). Although we have not compared intrathymic versus intravenous injection of this stem-cell-rich preparation, if passage through the thymus is either necessary or beneficial for induction of tolerance and only a fraction of peripherally infused cells reach the thymus due to host clearance mechanisms (47), then direct implantation of tolerogens into the thymus may allow a much lower dose of cells to accomplish the same result. Since host animals were not sacrificed but rather returned to the primate colony in the interests of animal conservation, we cannot answer whether microchimerism persisted within the thymus in these animals.

Recently, it has been suggested that immature forms of dendritic cells (18, 48), resembling those found within the thymus, may be important for the generation of donor-specific hyporesponsiveness following intravenous infusion of donor marrow cells. Such immature cell types are part of the progeny produced by differentiation of $CD34^+$ stem cells, supporting the con-

cept that intrathymically engrafted CD34+ cells may provide a clinically achievable means to ensure persistence of tolerance.

In comparison to these studies using CD34+ cells as intrathymic implants, previous studies using mature hematopoietic cells as intrathymic tolerogens have not reported prolonged peripheral blood microchimerism. The dependence of several rodent models on the presence of the graft for maintenance of tolerance (30, 32–33) suggests that a persisting source of foreign antigen, in either a central or peripheral location, might be necessary for such tolerance. The stem cell-enriched CD34+ cell fraction, by engraftment, might provide a self-perpetuating, and thus more permanent, source of donor antigens than mature leukocytes or allogeneic peptides (25–30), with or without an organ graft.

Skin grafts are usually considered a more difficult model of graft acceptance than cardiac grafts (49, 50). Therefore, the two- to threefold increase in skin graft survival time in marrow cell recipients for both primary donor allografts and one xenograft, as compared to third party grafts, would suggest that donor solid organ graft survival might be similarly prolonged. Of interest, donor cells were detected more frequently and skin graft survival prolonged in the xenograft recipient that did not receive immunosuppression. Taken together, these findings would support the premise that the presence of chimerism or, at least, donor antigens, may influence graft survival. This study does not resolve the current controversy over the significance of microchimerism (51, 52), but our findings do suggest that certain conditions may be found under which stem cell engraftment could occur without recipient conditioning (53), even in primates.

Second skin grafts in all allograft and xenograft skin graft recipients resulted in rejection, suggesting that the degree of tolerance or anergy produced was insufficient to overcome the addition of a new donor antigen load with its attendant antigen-presenting cells. Plausible explanations for the shorter survival of the second grafts in this model would include the local intragraft cytokine induction which reversed T-cell anergy (54, 56), the direct presentation of antigens by donor antigen-presenting cells in the skin graft (57, 60), or host sensitization to skin-specific non-MHC antigens not tolerized by hematopoeitic cells (49, 50), all of which have been shown to break tolerance in other models. Also, the larger size of the second grafts, designed to reduce recipient ingrowth into the graft, may have heightened the rejection response, as in Medawar's classical experiments in rabbits (61).

That said, in the second set of allografts, the grafts from the allogeneic marrow cell donor still survived longer than those from third party controls. Intermediate prolongation of second set skin grafts has been previously reported in a rodent model following intrathymic injections of xenogeneic donor splenocytes in combination with anti-lymphocyte serum and rapamycin (57).

Further characterization of both the xeno- and alloantibodies as to their specificities and cytotoxic potential would be needed to resolve their significance. Modulations of IgG alloantibody production have been reported after intrathymic injections of donor splenocytes in rodents (62) and after infusion of donor marrow in ALG-treated, class II-matched primates (63). In this series, two allogeneic marrow cell recipients did not mount detectable IgM responses following skin grafting. Although early and transient IgM production could have been missed, another explanation for this apparent lack of an IgM response would be a similar immunomodulatory effect on B cells.

In summary, the intrathymic implantation of CD34+ marrow cells provides a safe, simple means of achieving hematopoietic microchimerism with donor cells and prolonged donor skin allograft survival in this infant primate model. This method may be useful in the generation of postnatal tolerance to solid organ grafts, either with or without immunosuppression. The current protocol could have early clinical applicability in living related solid organ transplantation, such as parent-to-child lung transplants for cystic fibrosis, where the thymus is easily accessible within the operative field. Similarly, if simultaneous or subsequent transplantation of allografts and donor CD34+ marrow fractions results in the same auspicious results as seen in this sequential protocol, then applications in cadaveric organ transplantion could be pursued. Instillation of CD34+ marrow fractions into the thymus by thoracoscopy (64), mediastinoscopy, or percutaneous CT guidance could widen the applications to recipients of extrathoracic solid organ transplants, either living-related or cadaveric. Finally, since the technique produces hematopoietic chimerism without immunosuppression, extensions of this technique could have clinical applications as a benign alternative to myeloablative bone marrow transplantation for the treatment of genetic deficiency diseases of infancy, especially if, in the future, the number of circulating normal donor cells could be increased by stem cell growth factors or if the production of deficient proteins were enhanced by genetic engineering of introduced donor cells.

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